Controlling the Nanoscopic Confinement of Enzymes Inside ClyA Nanopores for Single-Protein Studies

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resolution has led not only to newly resolved intermediates and pathways, but also to new dynamics between states. For example, the number of detected intermediates in a pair of transmembrane helices increased from 2 to 14, and helix segments were observed to refold against high forces (F=60-140 pN) at standard pulling rates (v=0.1-5 μm/s). True equilibrium measurements characterized the extraction and insertion of small sections of different transmembrane helices. Cooperative folding and unfolding of a 3-amino-acid structural element was resolved in <15 μs at the C-terminal end of helix E. The transition's underlying energy landscape was deduced using a recently developed reconstruction method based on p_{fold} analysis, from which the water-to-bilayer folding free energy of hydrophobic helices were obtained. Similar back-and-forth transitions were observed within a portion of the folding pathway of helix A. These observations provide direct evidence that transmembrane helix segments can dynamically equilibrate between the aqueous and membrane phases, which has been proposed to play important roles in helix cotranslational integration via translocon. Our results demonstrate a new regime in studies of protein-folding dynamics: equilibrium folding and unfolding at 1-μs resolution. Moreover, we applied this technique to the important problem of measuring a membrane protein fold into its native membrane environment.

2552-Plat

Binding Mechanism of Purine Nucleotides to Mitochondrial Uncoupling Proteins Explored by Recognition Force Spectroscopy

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Regulated transport of protons across the inner mitochondrial membrane is essential for physiological processes such as ATP synthesis and heat production. Besides the involvement in thermogenesis, uncoupling protein family members (UCP1-UCP3) were proposed to regulate reactive oxygen species (ROS) and cell metabolism [1]. Although it is accepted that UCP activity is inhibited by purine nucleotides, the exact molecular mechanism of inhibition and binding is still unclear. Previously, we hypothesized that PNs bind to UCP1 from cis- and trans-side, although only cis-binding led to protein inhibition [2]. In this work we aimed to elucidate the binding of various nucleotides to UCP1-UCP3 on the single-molecule level. For this we reconstituted UCPs in bilayer lipid membranes at low density and probed the interaction forces between UCP and PNs coupled to AFM cantilevers using recognition force spectroscopy [3]. Our studies revealed that the life time of the UCP-PN bond tended to increase with the phosphorylation degree of the nucleotide. However comparison between UCP1, UCP2 and UCP3 revealed different strengths of binding. Moreover, mutations of three arginine residues (R276L, R83T, R182Q) known to be crucial for nucleotide binding were studied to reveal a detailed picture about the binding mechanism of the nucleotides to the UCPs on a structural level.

1) Rupprecht et al., PLOS One (2014) 9 (2):e88474.
2) Zhu et al., JACS (2013) 135, 3640.
3) Koehler et al., Biophysical Journal 106.2 (2014): 223a.

Platform: Biosensors

2553-Plat

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Ionic current recordings using biological nanopores provide a sensitive platform for monitoring reactions at the single-molecule level. Here we show that single enzymes can be trapped inside the confined space of a Cytolysin A (ClyA) nanopore for extensive periods of time by the electroosmotic flow, without the need of complex immobilization strategies or covalent chemistry. Remarkably, the binding of ligands and conformational changes to the internalized proteins are mirrored by specific changes to the nanopore conductance, indicating that ClyA nanopores are amenable for single-molecule enzymology studies. However, certain enzymes escape the nanopore too quickly to be properly sampled. Here we show that the residence time of proteins inside the ClyA lumen can be controlled by preparing interlocked protein-nanopore rotaxanes. Alternatively, the electrophoretic and electroosmotic driving forces can be fine-tuned by the precise modulation of the charges of the internalized proteins. The immobilization of single proteins inside the ClyA lumen opens new possibilities for real-time and label-free enzymology or proteomics studies, and for the fabrication of low-cost and portable sensing devices for the detection of analytes with high specificity.

2554-Plat

Label-Free Optical Biosensor Based on Photonic Crystal Surface Waves Reveals Binding Kinetics of Antibodies to Living Bacteria in Real Time

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Nowadays almost 20% of prescribed drugs lack a known mechanism of action resulting in no therapeutic but various side effects [1]. Efficiency of drug development can be significantly improved by knowing the drug - cell membrane binding kinetics data obtained in the real time and under the conditions as close as possible to the native ones. Unfortunately, label-free experiments with living bacteria performed using standard optical biosensors based on surface plasmon resonance are unable to deliver such information because of insufficient penetration depth of the evanescent field into an external media. Biosensors based on photonic crystal surface waves [2] overcome this drawback offering a solution for sensing of binding interactions with living "sufficiently thick" bacterial and eukaryotic cells. Besides that, simultaneous excitation of both s- and p-polarized waves is realized here enabling unambiguous separation of surface and volume effects.

As a demonstration of these possibilities, we conducted real-time experiments on binding kinetics of mono- and polyclonal antibodies against lipopolysaccharides to entire living bacterial cells E. coli. A chitosan-based protocol of biochip chemical functionalization for immobilization of bacteria has been elaborated. Data analysis reveals specific and non-specific binding, permitting calculation of binding constants and binding model verification. In many instances, our results enable to verify the data obtained earlier on model systems (e.g. cell fragments) elsewhere. We believe that real-time qualitative kinetic information collected under native cell environment will accelerate and optimize screening of drug candidates, and it will be used simultaneously with a viability test in the future.

1. Gregori-Puigjané, E. et al. Proc. Natl. Acad. Sci. U.S.A. 109, 11178 (2012).
2. Konopsky, V. N. et al. Sensors 13, 2566 (2013).

2555-Plat

Single Wavelength Excitation Dual Color Flim for Multiplexing Genetically Encoded FRET Biosensors

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Genetically encoded Förster Resonance Energy Transfert (FRET) biosensors are powerful tools for monitoring spatiotemporal biochemical activities in living samples. By labeling a probe protein with a pair of fluorescent proteins, FRET measurement allows to follow a conformational change of the probe sensor to a specific activity. A very exciting challenge is to follow two FRET biosensors at the same time in the same sample and in the same cellular compartment. But the multiplex approach suffers from two limitations: (i) a spectral bleed-through of the first acceptor in the second donor emission band that depends on the concentration of the two biosensors and (ii) the multiple excitation wavelengths which necessitates sequential acquisition that is not adequate to follow fast signal changes in highly dynamic biochemical activities.

Here, we report a method alleviating from both limitations. Taking advantage of the long stoke shift of LSSmOrange, we have used 440 nm single excitation wavelength of the two donor mTFP1 and LSSmOrange and a dual color FLIM to simultaneously measure two genetically encoded FRET biosensors. Moreover, thanks to the non-fluorescent acceptor sREACh for mTFP1 and of red-shifted mKate2 for LSSmOrange, we were able to neglect any spectral bleed-trough. With a dual spectral FLIM system we were able to detect fluorescence lifetime images of mTFP1 and LSSmOrange simultaneously and in the same cellular localization. We validated our approach by applying this methodology to simultaneously determine ERK and PKA activation in the same Hela cell using EKAR2G and AKAR4 biosensors respectively modified with mTFP1/sREACh and LSSmOrange/mKate2 pairs. By activating PKA with forskolin, the ERK pathway is not activated as expected. But surprisingly, by activating ERK with EGF, PKA is also activated denoting a cross talk between these two signaling pathway in Hela cell line.

2556-Plat

High-Resolution Analysis of Molecular Oxygen in Mammalian Cell Models by Phosphorescence Lifetime Imaging Microscopy

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